

REVIEW

Relations and interactions between cranial mesoderm and neural crest populations

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Abstract

The embryonic head is populated by two robust mesenchymal populations, paraxial mesoderm and neural crest cells. Although the developmental histories of each are distinct and separate, they quickly establish intimate relations that are variably important for the histogenesis and morphogenesis of musculoskeletal components of the calvaria, midface and branchial regions. This review will focus first on the genesis and organization within nascent mesodermal and crest populations, emphasizing interactions that probably initiate or augment the establishment of lineages within each. The principal goal is an analysis of the interactions between crest and mesoderm populations, from their first contacts through their concerted movements into peripheral domains, particularly the branchial arches, and continuing to stages at which both the differentiation and the integrated three-dimensional assembly of vascular, connective and muscular tissues is evident. Current views on unresolved or contentious issues, including the relevance of head somitomeres, the processes by which crest cells change locations and constancy of cell–cell relations at the crest–mesoderm interface, are addressed.

Key words angiogenesis; chick embryo; craniofacial development; muscle development; neural crest; paraxial mesoderm.

Introduction: head mesenchymal populations

Structures common to the head region of all vertebrates include segmentally organized dorsal (neural) and ventral (pharyngeal) epithelial tubes that are largely surrounded by mesenchymal populations called paraxial and lateral mesoderm, respectively. These mesenchymes generate the musculoskeletal structures necessary to protect the brain, provide sources of cardiovascular tissues and, ancestrally, formed structures necessary to facilitate the inflow of food and oxygen-bearing fluid (Gans & Northcutt, 1983).

During vertebrate evolution, cephalic paraxial mesoderm (Fig. 1) has retained close spatial relations to the central nervous system, and remains the principal

contributor to the neurocranium. Other parts of the mesoderm-derived head skeleton have undergone substantial evolutionary modifications, especially related to caudal head skeletal tissues. Contributions by cephalic lateral mesoderm in early vertebrates and their ancestors are not as well defined, owing to the absence both of clear demarcations between paraxial and lateral mesoderms and of lineage tracing data in multiple families. In amniotes, there are no known structural contributions by lateral mesoderm, exclusive of cardiovascular tissues, rostral to the laryngeal region.

Prechordal mesoderm is a variably sized mesenchymal population located between endodermal and neurectodermal epithelia immediately rostral to the notochord. In amniotes, this population becomes continuous laterally with paraxial mesoderm (Meier, 1982), but this contiguity is only established after the intra-embryonic expansion of both populations during gastrulation is completed (Kinder et al. 2001). In mice prechordal cells are cytologically distinguished from paraxial mesoderm by being ciliated (Sulik et al. 1994).

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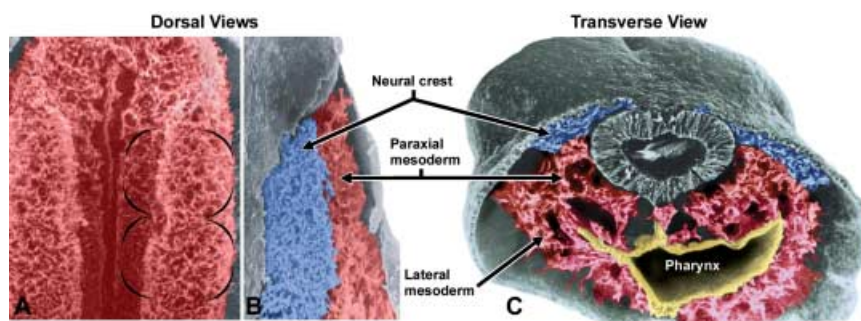


Fig. 1 Scanning electron micrographs showing the initial spatial relations among avian cephalic mesenchymal and epithelial tissues. (A) Dorsal view of a stage 8, four-somite embryo in which the surface ectoderm and neural tube have been removed, exposing underlying paraxial mesoderm (red). The brackets indicate the boundaries of somitomeres 2 and 3, as proposed by Meier (1979). (B) Comparable view of an embryo approximately 10 h more advanced (ten somites), with the neural tube in place (visible to the left). The leading edge of the neural crest (blue) partially overlies paraxial mesoderm. In C, a ten-somite embryo was transected at the level of the midbrain to show the early interface between crest and paraxial mesoderm cells. A, B, colour added from archival prints provided by S. Meier; original SEMs published in Meier (1979) and Anderson & Meier (1981); C, colour added from an original micrograph provided by K. Tosney (1982).

Prechordal mesoderm cells are key participants in ventralization and bilateralization of the prosencephalon (Shimamura & Rubenstein, 1997; Anderson et al. 2002), and express genes related to this function that further distinguish them from paraxial mesoderm (Camus et al. 2000; Withington et al. 2001).

A hallmark of vertebrates is the augmentation of all these mesoderm populations by secondary mesenchymal cells derived from the neural crest, which forms at the neural plate–surface ectoderm border before or shortly after neural tube closure (Selleck & Bronner-Fraser, 1995; Kulesa et al. 2004). Along most of the body axis, neural crest cells form peripheral sensory and autonomic neurons, glial and pigment cells, some of which are presaged in urochordate embryos (Jeffery et al. 2004). The evolution of vertebrates was critically dependent upon the formation and modification of jaws and gills, all of which were generated by the addition of multipotential cells including progenitors for a wide range of loose and dense connective tissues, perivascular smooth muscles, and a variety of secretory cells (Hall & Hörstadius, 1988; Hall, 1999; Le Douarin & Kalcheim, 1999) to the neural crest repertoire. Although primarily restricted to the head region in extant vertebrates, the possibility that trunk crest cells also contributed to exoskeletal tissues in extinct groups, e.g. ostracoderms, remains actively debated (Northcutt & Gans, 1983; Wada, 2001; Graham et al. 2003; Kuratani, 2005).

Generating neural crest cells with competence to form craniofacial connective tissue lineages was, by itself, of little evolutionary benefit. Multiple peripheral tissues needed to acquire the capability of producing

skeletogenic inducing signals that are spatially and temporally coordinated with the presence of neural crest cells in branchial (Northcutt, 1990) and midfacial (Helms et al. 2005) regions. Irrespective of their embryonic origin, precursors of connective tissues progressively acquire, display and impose upon their neighbours the anatomical patterning unique to musculoskeletal assemblies in each part of the organism. Thus, in addition to the preceding histogenic activities taking place within and around newly emergent neural crest cells, the ability to generate skeletal structures and assemblies unique to each part of the midface and branchial regions needed to be programmed into neural crest cells and integrated with their surroundings. The origins and confluence of these independent yet obligatorily integrated ontogenetic processes represent a remarkable and still poorly understood feature of early vertebrate evolution.

The scope of this review is deliberately inclusive with respect to craniofacial mesenchymal populations. Although each has unique embryonic histories and properties, development of peripheral components of the head is a collective enterprise requiring interactions among as well as contributions by all these populations. Our emphasis is on early paraxial mesoderm – neural crest relations and interactions, primarily at the hindbrain and branchial arch regions of the head. Critical roles for other signalling domains, e.g. the central nervous system and pharyngeal epithelia, and examinations of later stages as well as other regions of facial and skull development are covered elsewhere in this volume.

Formation of head mesoderm populations

Gastrulation establishes a trilaminar embryo with the middle layer, mesoderm, arising by the involution and delamination of cells from the ectoderm (epiblast) layer at the primitive streak. In the mouse, cells within the caudal epiblast of the early primitive streak are the first to undergo localized epithelial to mesenchymal transformation (Sulik et al. 1994). This population remains in a median position and, concomitant with precursors of the rostral neural plate and underlying endoderm, expands rostrally (anteriorly) to form the mesenchymal component of the prechordal plate (Lawson, 1999; Camus et al. 2000).

Subsequent transformations at or immediately caudal to the primitive node, which is the cranial pole of the streak, establish progenitors of intra-embryonic paraxial and lateral mesoderm tissues, including some cardiac progenitors (Smith et al. 1994; Psychoyos & Stern, 1996; Kinder et al. 2000). These spread rostrally as a broad, medio-lateral sheet, but remain separate from prechordal mesoderm until their intra-embryonic expansion is completed.

The boundary between paraxial and lateral mesoderm populations is indistinct in the head region of amniotes and, in the absence of molecular identifiers, is arbitrarily placed lateral to the dorsal margin of the pharynx. In contrast to many anamniote embryos in which parts of a serosa-lined body cavity extend into the head (Goodrich, 1930), and thus permit identification of splanchnic and somatic lateral mesoderms, avian and mammalian embryos undergo precocious head folding before these landmarks are present. Although there have been descriptions of isolated serosa-lined head cavities in amniotes (Wedin, 1953; Gilbert, 1957), these have not been verified by more recent examinations. It is not until the level of the occipital somites, adjacent to the caudal aspect of the pharynx, that definitive coelomic (future pleuropericardial) spaces and also aggregation of intermediate mesodermal cords are present.

Spatial organization of cephalic paraxial mesoderm

Paraxial mesoderm is generated as a mesenchymal population over a period of several days as the primitive streak regresses and generates progressively more caudal structures. However, beginning beside the hind-

brain and caudal to the otic vesicle, paraxial mesoderm cells do not remain mesenchymal. Eight to 18 h after their formation, depending on the species and axial location, these mesenchymal cell populations cyclically form segregated, epithelial somites on each side of the neural tube. In chick embryos a new somite is formed approximately every 90 min, whereas in mice this process typically requires close to 2 h. The medial and lateral parts of each somite arise from mesenchymal progenitors that trace their origins to distinct, separate sites of involution at or caudal to the primitive node (Tam et al. 2000; Freitas et al. 2001). Whether this is also true for head paraxial mesoderm is not known.

The precise, periodic formation of somites requires two concurrent processes: a caudal-to-cranial diminishing gradient of growth factors, especially fibroblast growth factor (FGF), and a molecular oscillator, the segmentation clock, which utilizes a periodic expression of Notch pathway-related genes such as *hairy1* and *hairy2* or *lunatic fringe* in the presomitic mesoderm (Jouve et al. 2002). In chick embryos, this dynamic pattern of cyclical gene expression begins with the initial formation of paraxial mesoderm cells, and for most of the body each cycle corresponds to the subsequent genesis of one additional somite. Cranial mesoderm exhibits two distinct pulses of cyclical gene expression, but does not condense to form somites. It has been proposed that two regionalized domains may still exist in head paraxial mesoderm (Jouve et al. 2002); however, no known morphological outcome matches this pattern.

The issue of 'head segmentation' has been one of the most controversial and divisive (pun intended) topics in comparative vertebrate anatomy (reviewed by Neal, 1918; Romer, 1972; Jarvik, 1980; Kuratani, 2005). Although fossil data on branchial skeletal structures and evolutionary data on cranial nerves are richly available, the historical record of most head mesoderm-derived structures is incomplete and interpretations often speculative. In extant vertebrates the muscles and skeletal elements formed by mesodermal populations are often highly derived and frequently bear scant identifiable relation to their progenitors, making identification of embryonic and phylogenetic antecedents impossible in the absence of direct, prospective lineage tracing studies.

Classic models of head segmentation postulated that head mesoderm is, like the brain (Vaage, 1969), organized into metameric units, albeit morphologically undetectable in amniotes. These cryptic units correspond to a proposed complete segmental series of motor

nerves emerging from the brain beginning at the midbrain (oculomotor) level (reviewed by Romer, 1972; Northcutt, 1990). The absence of a fully matched metameric array in any single vertebrate group was attributed to secondary loss (Goodrich, 1930; Jarvik, 1980).

Stephen Meier (1979) was the first to discover evidence of a metameric organization within cephalic paraxial mesoderm. Following removal of overlying neural and surface ectoderm, Meier used stereo-SEM to examine the superficial surface of this mesoderm in chick embryos. He observed periodic domains in which mesenchymal cells together with their filopodial and lamellipodial processes were aligned in concentric arrays around a central site. Meier termed these domains somitomeres (brackets, Fig. 1A). The borders between adjacent somitomeres were readily apparent because the outermost concentric array of cells of each curved away from that of its neighbour, creating a shallow groove. This pattern could be detected during the early neural plate stage, when paraxial mesoderm is largely overlain by neuroepithelium, and is maintained until after neural tube closure, by which time both neural and non-neural surface ectoderm abut somitomeric mesoderm.

The discovery of domains with a similar appearance in caudal, presomitic paraxial mesoderm (i.e. segmental plate, Packard & Meier, 1983) prompted the suggestion that somitomeres represent a morphological stage en route to somite formation, and that head mesoderm has evolutionarily become arrested in this process (Jacobson, 1993). Indeed, epithelial clusters resembling somites have been found rostral to the otic vesicle in several anamniote species, e.g. *Xenopus* (Chung et al. 1989), and in many cases were touted as the precursors of extraocular muscles (Adelmann, 1927; Edgeworth, 1935).

Since Meier's original observations, head somitomeres have also been described in quail (Meier, 1982), mouse (Meier & Tam, 1982; Tam & Meier, 1982), snapping turtle (Meier & Packard, 1984; Packard & Meier, 1984), shark, newt (Jacobson & Meier, 1984) and medaka (Martindale et al. 1987) embryos, and in some fishes they are visible upon gross dissection. In amniote and medaka embryos, cranial paraxial mesoderm is subdivided into seven somitomeres, whereas only four cranial domains have been found in the other species listed above (Jacobson, 1993). In chick embryos the first pair of somitomeres is initially lateral to prechordal mesoderm, beneath the mid-prosencephalon, but shifts caudally during head flexure and optic vesicle evagination. The mesencephalon is flanked by somitomeres 2

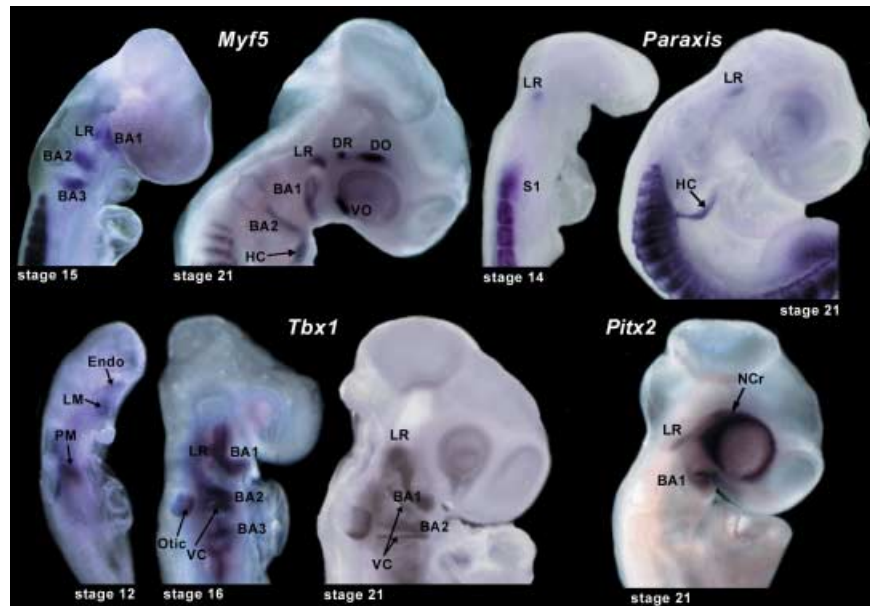
and 3, the isthmus and metencephalon by somitomere 4, with the remaining somitomeres located beside the myelencephalon. There are discrepancies between mouse and chick embryos in the alignment of specific somitomeres with neuromeric boundaries, but it is not known whether these reflect different primary patterns or secondary dislocations owing to differential elongation of the rostral neural tube (Gilland & Baker, 1993). Also, the first true somite is generated further caudal to the otic vesicle in mammalian embryos.

Both the existence and the developmental significance of cranial somitomeres are topics of significant debate. More recent attempts to identify them using comparable methods have not been successful (Wachtler & Jacob, 1986), and different results are obtained depending upon how the overlying ectoderm and extracellular matrix are removed (Jacobson, 1988). It is important to keep in mind that the only identifying features of somitomeres are those on the superficial surface of paraxial mesoderm; this may be evidence of a metameric pattern but is not synonymous with segmentation.

No other morphological features apart from the alignment of the cell somas and their processes have been found that may indicate the presence of intersomitomeric discontinuities or boundaries at deeper levels. An analysis of packing densities within the cranial mesenchyme has failed to reveal any segmental periodicity (Freund et al. 1996). Lineage tracing of mesoderm cells in adjacent somitomere territories using distinct fluorescent dyes demonstrates that somitomeres are not units of lineage restriction and do not define compartments whose cells have distinct spatial properties (Trainor et al. 1994), as do somites and rhombomeres (Fraser et al. 1990).

Compared with other prospective or actually segmented tissues in the embryo, such as the forebrain, hindbrain, segmental plate or somites, there is a relative dearth of information concerning the patterns of gene expression within cranial paraxial mesoderm, and what information is available has yet to reinforce or refute the somitomere model. Several early markers of somites, e.g. *Paraxis*, *Pax3*, *Lbx1* (Delfini & Duprez, 2000; Gross et al. 2000) are present in head paraxial mesoderm but show no evidence of segmental expression (Fig. 2). *Paraxis*, which encodes for a basic helix loop helix transcription factor, is expressed in newly formed paraxial mesoderm in mouse embryos, extending rostrally to the level of otic sulcus beside the junction

Fig. 2 *In situ* hybridizations performed on intact chick embryos to show sites of gene expression for the transcription factors *Myf5*, *Paraxis*, *Tbx1* and *Pitx2*. *Myf5* is activated in all skeletal muscle precursors, whereas *Paraxis* is limited to somitic cells plus the lateral rectus precursor. Both *Tbx1* and *Pitx2* show complex and stage-specific patterns of expression in cephalic mesenchymal and epithelial cells. These briefly are expressed in subsets of head muscles. BA1, 2, 3: branchial arches; HC, hypoglossal cord; LM, PM: lateral and paraxial mesoderm; DR, LR: dorsal and lateral rectus muscles; DO, VO: dorsal and ventral oblique muscles; S1, 1st somite; VC, visceral cleft ectoderm.



of rhombomeres 5 and 6 (Blanan et al. 1995). In the chick, *Paraxis* transcripts are found in all somites, but only in the most caudal aspect of unsegmented head paraxial mesoderm and in the lateral rectus primordium (Fig. 2; Mootoosamy & Dietrich, 2002; Borue & Noden, 2004). Several genes expressed in subsets of somitic cells, e.g. *Pax1* and *Scleraxis*, are not expressed in head mesoderm, although the lineages associated with these genes differentiate in both somitic and unsegmented head mesoderms.

Expression patterns of other genes have been interpreted as evidence for regionalization within cranial paraxial mesoderm prior to the onset of neural crest cell migration. In early somite stage mouse and chick embryos, the homeobox gene *Hoxb-1* is transiently expressed in the cranial paraxial mesoderm up to the level of the preotic sulcus, where the junction between rhombomeres 2 and 3 will later form (Frohman et al. 1990). These data together with those describing mouse *Paraxis* expression might designate three regions within cranial mesoderm, a rostral domain beside the midbrain, spanning somitomeres 1–3, an intermediate domain lateral to the metencephalon (somitomeres 4 and 5), and a caudal domain extending to the first somite.

However, caution must be exercised in extrapolating from these data to postulate a metameric or other pre-pattern within head paraxial mesoderm (Stone & Hall, 2004). *Hoxb-1* may be facilitating retinoic acid responses in caudal head mesoderm, and to date no abnormalities in head structures are associated with loss of *Paraxis*

function. Also, it is evident that many regulatory genes, e.g. *Tbx1*, are activated in waves that move through regions of cranial paraxial mesoderm (Fig. 2). These expression sites may, briefly, coincide with a location or boundary that, based on mapping data, has some developmental significance. Such congruency, no matter how transient, inevitably appeals to our bias to align molecular, cellular and anatomical boundaries. However, given the paucity of genes yet identified in head mesoderm it is premature to elevate these correlations to causal status. Many growth factors, e.g. sonic hedgehog, FGFs, bone morphogenetic proteins (BMPs), are released from multiple tissues adjacent to head paraxial mesoderm at several sites and times (Crump et al. 2001, Moore-Scott & Manley, 2005), but except for those associated with pharyngeal pouches and placodes there are none that show periodic expression sites. Thus, at present the existence of a metameric pattern within cranial paraxial mesoderm is unsubstantiated, and the significance, if any, of somitomeres remains unresolved.

Movements and differentiation within head mesoderm: angiogenesis and myogenesis

Paraxial mesoderm in the head generates the same cell types as do somites, albeit often in different proportions and with a different schedule. Both give rise to angioblasts and hemangioblasts (which often are indistinguishable at early stages), smooth and skeletal myogenic

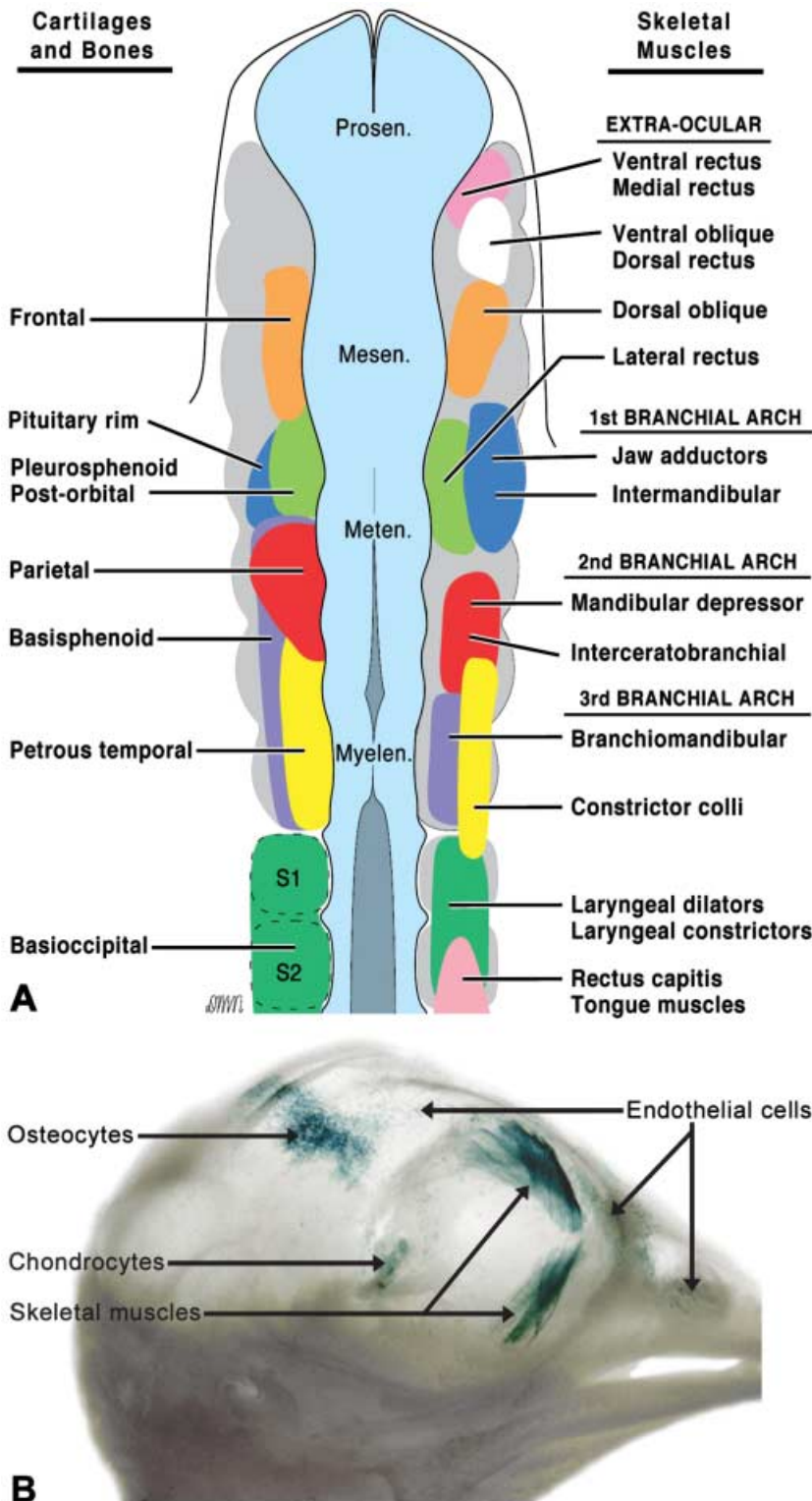


Fig. 3 (A) Summary map showing the sites of origin of muscles (right side) and skeletal elements (left) derived from chick paraxial mesoderm. (B) A 15-day chick embryo that received an injection of *LacZ*-bearing retroviruses beside the midbrain 2 weeks previously. Chondrogenic cells have remained stationary and are within the postorbital cartilage, whereas osteogenic cells have moved dorsally and, in this case, contributed to the frontal bone. Cells in myogenic and angiogenic lineages exhibit additional distinct behaviours, taking each population to different locations in the head. After Evans and Noden (2005).

cells, and a variety of loose and dense connective tissues (Noden, 1988, 1991b; Couly et al. 1992; Evans & Noden, 2005). The sites of origin of mesoderm-derived craniofacial skeletal elements and muscles in avian embryos are illustrated in Fig. 3(A).

A striking feature of cranial paraxial mesoderm is the diversity of movements exhibited by each of the lineages that arise therein. Chondrogenic cells initiate and complete their differentiation without significant movement (Fig. 3B). As is the case for sclerotomal cells

in the trunk, differential growth creates elongated tongues and sheets of cartilage, but there is little or no cell movement preceding chondrogenesis. Later, these same populations participate in endochondral ossification of the walls and floor of the neurocranium. By contrast, mesodermal progenitors of intramembranous bone move dorsally around the brain, occupying sites lateral and dorsal to the neural tube prior to their overt differentiation as osteocytes. Precursors of skeletal muscles aggregate close to their sites of origin within paraxial mesoderm, and concomitantly activate muscle-specific transcription factors. The primordia of each muscle or muscle group, e.g. first arch myoblasts, subsequently move as cohorts into branchial or periorcular regions. Their interactions en route with neural crest cells will be discussed later.

Embryonic angioblasts migrate invasively in all directions (Figs 3B and 4). This invasive behaviour, which is unique to a subset of endothelial precursors in vertebrate embryos, is accompanied by a rapid increase in the number of angioblasts. Following this burst of cell migrations, angioblasts cease moving and establish close apposition with other nearby endothelial precursors, forming vesicles that fuse and cords that remodel as early embryonic blood vessels (Noden, 1989, 1991a).

These mesoderm cell tracing studies indicate that early in development, often before there is any overt display of lineage-specific markers, cells committed to each major derivative within head paraxial mesoderm exhibit strikingly different behaviours. At present, there are no molecular data that explain stationary behaviour (cartilage) or movements in a planar manner (calvarial bone), or as cohorts (skeletal muscle). Surprising, even the aggressively invasive behaviour of embryonic angioblasts has escaped biochemical analysis.

Lineage determination within trunk paraxial mesoderm and its integration with somitogenesis have been well studied in many species (reviewed by Dockter & Ordahl, 2000; Olivera-Martinez et al. 2004a; Scaal & Christ, 2004). Three general features that have emerged are (1) that whatever biases may arise prior to somite formation (Kiefer & Hauschka, 2001; Linker et al. 2003), neither the skeletogenic nor the myogenic lineages become stably determined *in vivo* until after the somitic epithelium formation is completed; (2) for both these lineages there exist consortia of signals from multiple sources, including other somite cells, that must act coordinately to induce their differentiation with proper timing and locations (Borycki & Emerson, 2000; Pownall

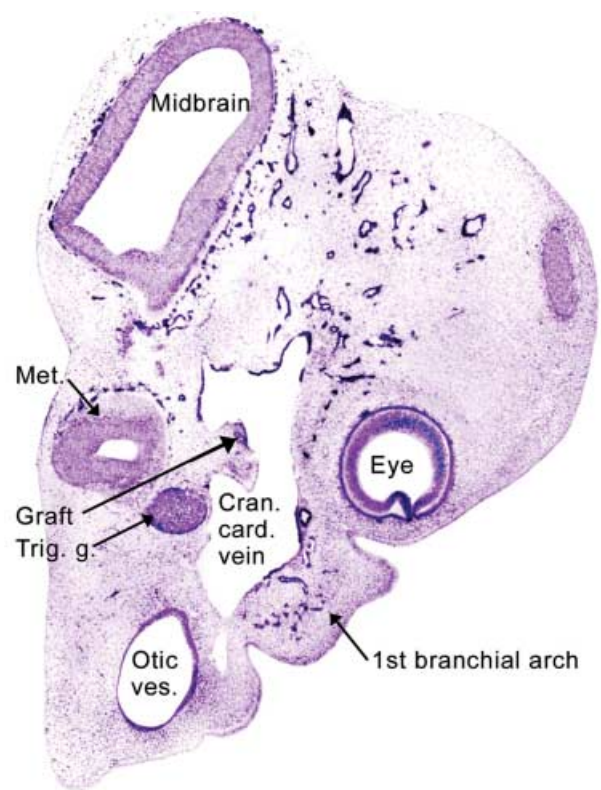


Fig. 4 The widespread, invasive migrations of angioblasts are illustrated by immunostaining for quail endothelial cells 60 h after implanting a small piece of quail mesoderm beside the metencephalon of a chick embryo. As is evident in this parasagittal section, angioblasts have moved in all directions from the site of implantation and contributed to both large (cranial cardinal vein) and small blood vessels, including precursors of meningeal and intraneural vessels. Met., metencephalon; Trig. g., trigeminal ganglion. From Noden (1991a).

et al. 2002; Buckingham et al. 2003); and (3) progressive emergence of some lineages, e.g. skeletal muscle, is spatially linked to discrete epithelial sites and boundaries within each somite (Ordahl & Le Douarin, 1992; Gros et al. 2004).

Angioblasts and hemangioblasts do not exhibit a similar, progressive determination, and indeed some are already established during gastrulation within segmental plate mesoderm (Drake & Fleming, 2000; Sato et al. 2002) and throughout head paraxial and lateral (but not prechordal) mesoderm (Noden, 1989, 1991a). Based on transplantation data and immunocytochemical identification of VEGF-R2(Kdr/Flk-2)-positive cells, endothelial precursors are fairly uniformly dispersed in mesoderm throughout the head. By the neurula stage, angioblasts constitute more than 30% of the paraxial mesoderm population and approach 100% in lateral

mesoderm associated with prospective branchial arch 1–3 locations.

Cranial mesoderm generates precursors of all voluntary muscles in the head (Noden, 1983b; Couly et al. 1992; Koentges & Lumsden, 1996). The primordia of extra-ocular and branchial arch muscles differ in their dorso-ventral and medio-lateral sites of initial appearance within paraxial mesoderm (Couly et al. 1992). Paraxial mesoderm populations located superficially beside the hindbrain are the sources of the branchial arch and associated musculature (Noden, 1983b; Schilling & Kimmel, 1994; Trainor et al. 1994). These include jaw closing (1st arch) and opening (2nd arch) muscles, facial muscles in mammals, and hyobranchial muscles innervated by the trigeminal, facial and glossopharyngeal nerves. This spatial organization is especially important in assessing the roles of neighbouring tissues, including the neural crest, as sources of myogenesis promoting or inhibiting factors.

Differentiating skeletal muscle is the best described lineage in cranial paraxial mesoderm, in large part because many myogenic cells activate lineage-specific transcription factors, e.g. *Myf5*, *MyoD*, *MyoR*, *Capsulin* (*pod1*), *Tbx1*, *Tlx1*, during or shortly after commitment. Myogenesis in head paraxial mesoderm lags behind myotome differentiation in somites, and generally branchial arch myogenesis precedes extra-ocular muscle determination (Fig. 2, *Myf5* series), more so in the mouse than in the chick embryo. Also, the period from onset of *Myf5/MyoD* expression to production of desmin and myosin proteins is greatly prolonged in cranial muscles, especially in comparison with trunk axial muscles (Hacker & Guthrie, 1998; Noden et al. 1999; Mootoosamy & Dietrich, 2002). The interplay among these early muscle regulatory factors is not well defined for head muscles. *MyoR* and *Capsulin* function cooperatively in regulating the initial steps of branchial skeletal muscle specification, as evidenced by loss of *MyoD* and *Myf5* expression in their combined absence (Lu et al. 2002).

Tbx1 is also necessary for the early activation or stabilization of *MyoD* and *Myf5* (Kelly et al. 2004). However, this gene is not unique to the myogenic lineage. It is expressed in paraxial mesoderm both rostral and caudal to sites of branchial arch and lateral rectus myogenesis, and is not found in other head muscles. These results suggest that *Tbx1* must act in concert with other myogenic factors that provide site specificity.

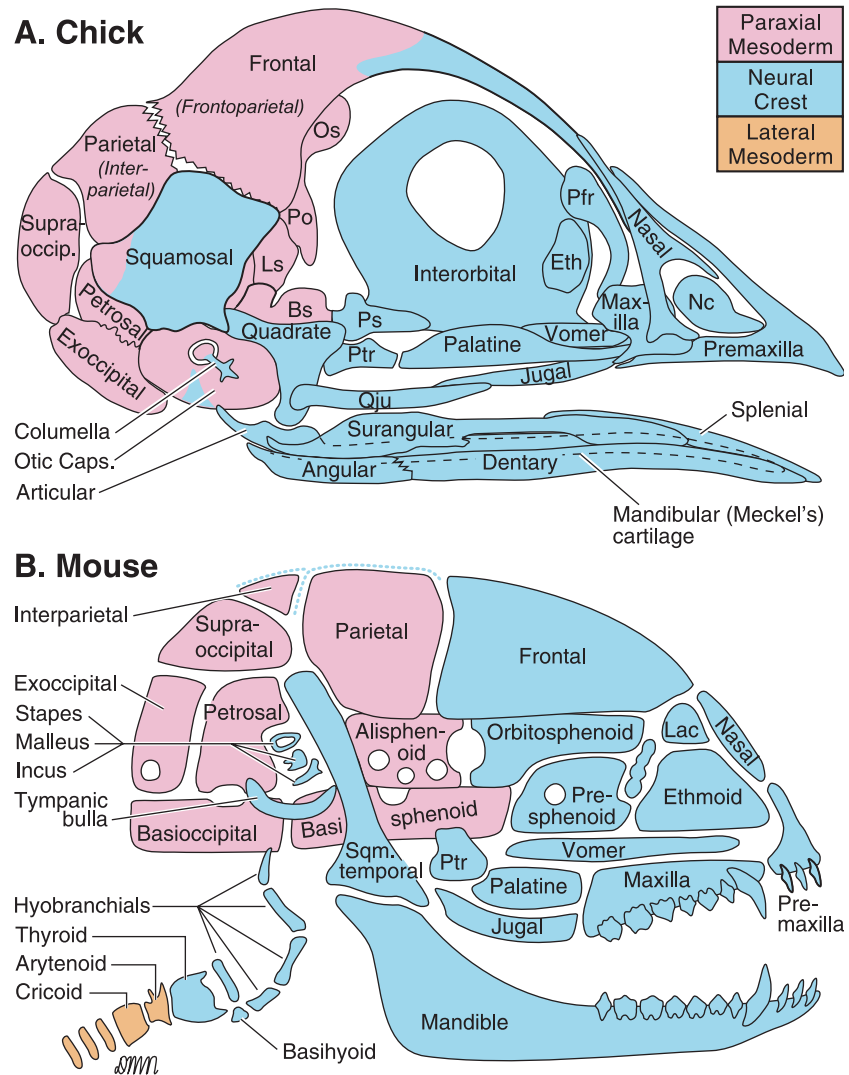
Extra-ocular muscles have a mixed origin. During bilateralization of the optic primordia and subsequent elevation of prosencephalic neural folds, prechordal

mesoderm cells are passively carried laterally then dorsally, where they become indistinguishable from the most rostral paraxial mesoderm. Later, as optic vesicles evaginate, these combined paraxial and prechordal mesodermal populations are displaced caudally, and occupy the sites from which extra-ocular muscles innervated by the oculomotor nerve arise (Wachtler et al. 1984). The primordia of dorsal oblique myoblasts arise caudal to these, in paraxial mesoderm located beside the mid-mesencephalon, a site they share in avian embryos, with precursors of the frontal bone osteoblasts and rostral neurocranial chondroblasts (Noden, 1991b; Jiang et al. 2002).

The common precursor population of lateral rectus and accessory ocular protective muscles (e.g. avian pyramidalis and quadratus nictitans, mammalian retractor bulbi) arises immediately adjacent to the ventro-lateral surface of the neural tube, in this case beneath rhombomeres 1 and 2 (future metencephalon; Wahl et al. 1994; Noden et al. 1999; Mootoosamy & Dietrich, 2002). Early in vertebrate evolution this population was co-opted from a branchial to an ocularotatory location and function (Edgeworth, 1935). The deep embryonic origin of the lateral rectus is unique among amniote muscles, and it may be analogous to adaxial myoblasts described in zebrafish (Hirsinger et al. 2004). In fish, these deep muscle progenitors are sonic hedgehog dependent. However, the lateral rectus primordia, like other cranial paraxial myogenic populations, lacks the patched receptors and is thus unresponsive to sonic hedgehog. Additionally, the lateral rectus primordia express several transcription factors that otherwise are unique to trunk (somite-derived) myoblasts, including *Paraxis* and *Lbx1* (Fig. 2; Mootoosamy & Dietrich, 2002; Borue & Noden, 2004).

All head muscle primordia exit from their initial niches in paraxial mesoderm and move into periocular and branchial locations populated by connective tissue progenitors derived from the neural crest (Noden, 1983a, 1986; Schilling & Kimmel, 1994; Trainor & Tam, 1995; Koentges & Lumsden, 1996). This contrasts with most trunk muscle primordia, which either remain contiguous with their neighbouring paraxial mesoderm cells (Williams & Ordahl, 2000; Burke & Nowicki, 2003; Nowicki et al. 2003) or are evoked to move as individual myoblasts into lateral mesoderm at appendicular (Hayashi & Ozawa, 1995; Brand-Saberi et al. 1996) and glossolaryngeal (Noden, 1983b; Huang et al. 1997) sites. The only exception among somite-derived muscles are

Fig. 5 Schematic chick and mouse skulls showing the contributions of neural crest, paraxial and lateral mesoderms to the cranial skeleton. The avian map is based on transplantation and retroviral lineage tracings in the chick embryo; hyobranchial structures, all of which are derived from neural crest cells, are not shown. The mouse map is based largely on the location of neural crest cells, as identified by expression of *LacZ* driven by a *Wnt1* promoter in *cre-lox* transgenic embryos (Jiang et al. 2002). Origins of mouse laryngeal cartilages are by extrapolation from avian data, with the caveat that birds do not have a thyroid cartilage. Blue dots indicate the locations of crest cells present at sites of calvarial sutures. Abbreviations (Figs 5, 9 and 11): Ang, angular; Art, articular; Bs, basisphenoid; Den, dentary; Eth, ethmoid; Lac, lacrimal; Ls, laterosphenoid*; Mc, mandibular cartilage; Nc, nasal capsule; Os, orbitosphenoid*; Pal, palatine; Pfr, prefrontal; Po, postorbital; Ps, presphenoid; Ptr, pterygoid; Qd, quadrate; Qju, quadratojugal; San, surangular; Sqm, squamosal; *regions of the pleurosphenoid.



progenitors of tongue muscles, which emigrate from somites 2–5 and move en masse as the hypoglossal cord (Fig. 2B) into the future tongue primordium, whose connective tissues are of neural crest origin. The significance of the interactions between mesoderm and neural crest cells during myogenesis is discussed below.

Movements and differentiation within head mesoderm: connective tissues

Later during embryonic development, cranial paraxial mesoderm produces cartilaginous and bony elements of the neurocranium, including the parietal, petrous and basisphenoid bones of the neurocranium (Fig. 5; chicks: Couly et al. 1992; mice: Jiang et al. 2002). Identifying the origins of calvarial elements has sparked

controversy (Noden, 1975, 1978a, 1983a; LeLievre, 1978; Couly et al. 1992) in two aspects: the precise locations of the boundary between crest- and mesoderm-derived osteogenic precursors, and the correct nomenclature of avian vs. mammalian skull elements. Differing results based on quail-chick transplantations centered around the ability to graft neural crest or mesoderm progenitors exclusive of contamination by the other, which becomes increasingly difficult at progressively more caudal regions of the hindbrain. However, the use of replication-incompetent retroviral constructs containing a stable reporter construct (Mikawa & Gourdie, 1996) has substantiated the dual origin of the avian frontal bone and exclusively mesodermal origin of the avian parietal bone (Evans & Noden, 2005; Figs 3B and 5). Later, as the cerebral vesicles expand beneath the

frontal and parietal elements, crest-derived meningeal cells surrounding these parts of the brain are brought beneath the frontal and parietal osteogenic tissues. These crest cells are, however, restricted to the dura and do not form periosteum or osteocytes.

Evidence that the mouse frontal bone is fully rather than partially (e.g. chick frontal) of neural crest origin (Jiang et al. 2002) raised speculation that the neural crest–mesoderm boundary might have shifted location in one (possibly both) of these vertebrate lineages. At a mechanistic level, this would imply that pattern-generating and osteogenic responsive properties unique to each of these mesenchymal populations would need to have adopted that of the other. Thus far, no evidence of such a programmatic shift has been identified, although the range of vertebrates in which it has been tested is limited (Schneider, 1999; Schneider & Helms, 2003; see also Gross & Hanken, 2005).

A more likely explanation is that the neural crest–mesoderm interface has remained constant relative to the brain and pharynx. The presence of multiple independent ossification centres in the skull roofing elements of anamniotes is well documented (Moore, 1981; de Beer, 1985), and skull bones of extant amniotes represent combinations of loss, fusion and differential expansion of these centres (Thompson, 1993). Each of the paired avian frontal bones arises by the fusion of two intra-membranous ossification centres (Jollie, 1981), with the more rostral centre being of neural crest origin (LeLievre, 1978; Noden, 1978) and the caudal centre of mesoderm origin. If these centres remain unfused, an outcome such as that in most (but not all, see Novacek, 1993) mammals would result.

Although great attention has been paid to identifying homologous bones in the viscerocranium (Jollie, 1981; Striedter & Northcutt, 1991) and the floor of the braincase (Presley, 1993), there has been less interest in and consensus about roofing elements (reviewed by Jarvik, 1980; Novacek, 1993). Most current investigations of the mammalian calvaria focus on sites and mechanisms of suture formation (Opperman, 2000), which is appropriate given their clinical significance in the genesis of craniosynostoses (reviewed by Ridgway & Weiner, 2004). Recognizing that embryonic origin is only one of several criteria used to establish homology (Wagner, 1989; Panchen, 1999; Müller, 2003), perhaps the avian frontal bone would more appropriately termed a 'frontoparietal' element, with the parietal becoming the interparietal bone.

Formation of neural crest cells

Neural crest induction is a multistep scenario whose antecedent processes begin during the delineation of the neural plate (reviewed by Meulemans & Bronner-Fraser, 2004). Crest cells are generated transiently along almost the entire axis in a zone called the neural plate border, which is located at the sharp interface of non-neural surface ectoderm and neural plate tissue (Moury & Jacobson, 1990; Dickinson et al. 1995; Selleck & Bronner-Fraser, 1995; Mancilla & Mayor, 1996). The appearance of crest cells or their immediate precursors is typically assayed by the expression of members of the *Snail* (*Snail* and *Slug*) family of zinc-finger transcription factors within border cells (Nieto et al. 1994; Mayor et al. 1995). Typically there is a narrow temporal window during which all neural crest cells form at each axial level; in avians and mice this period corresponds to approximately 8–12 h in the head, somewhat longer in the trunk (Tosney, 1978, 1982; Serbedzija et al. 1992; Basch et al. 2000, 2004; Ruffins & Bronner-Fraser, 2000).

The formation of neural crest cells requires planar interactions across this interface and also influences from underlying paraxial mesoderm (Selleck & Bronner-Fraser, 1995; Bonstein et al. 1998; Marchant et al. 1998), as was first postulated by Raven & Kloos (1945). In response to contact-mediated signals, cells at the neural plate border commit to undergo an epithelial to mesenchymal transition, although there is considerable variation between species and also at different axial levels in the delay between commitment to and execution of this transition (Knecht et al. 1995).

In studies of frog and fish embryos, trunk neural crest cells are specified at the border zone by a precise threshold within a graded concentration of members of the BMP family (Mayor et al. 1995; Morgan & Sargent, 1997; Tribulo et al. 2003). Surprisingly, it is the dorsolateral mesoderm in *Xenopus*, in contrast to the surface ectoderm in other species, that is capable of inducing neural crest cell formation in ectodermal explants (Marchant et al. 1998). This dorsolateral mesoderm produces either BMP inhibitors or a specific neural crest inducer, such that low, intermediate and high levels of BMP activity induce neural plate, neural crest and epidermal tissues, respectively.

Wnt signaling has also been postulated to be a key player in neural crest cell formation (Saint-Jeannet et al. 1997; Garcia-Castro et al. 2002). Sources of Wnt signals include the ectoderm in frog, avian and fish

embryos (*Wnt7b*, Chang & Hemmati-Brivanlou, 1998; *Wnt6*, Garcia-Castro et al. 2002; *Wnt8*, Lewis et al. 2004). However, intracellular Wnt antagonists designed to block specifically the response of border cells to Wnt signalling fail to inhibit the induction of neural crest cell markers by the paraxial mesoderm (Monsoro-Burq et al. 2003).

The interplay among Wnts and BMPs and their several antagonists/blockers, which are also present, is probably critical to the positioning and timing of neural crest formation, and possibly also to the early delineation of sublineages within crest populations (Sela-Donenfeld & Kalcheim, 1999, 2000; Trainor & Krumlauf, 2002). In *Xenopus*, the combination of BMP antagonists with members of the *Wnt* family such as *Wnt1* and *Wnt3a* (Saint-Jeannet et al. 1997), *Wnt7b* (Chang & Hemmati-Brivanlou, 1998) and *Wnt8* (LaBonne & Bronner-Fraser, 1998) is required to initiate neural crest specification.

FGF2 signalling in combination with attenuated BMP activity also has the ability to induce neural crest cell formation in *Xenopus* explant assays (Mayor et al. 1997; Villanueva et al. 2002), and over-expressing a dominant-negative FGF receptor (*Fgfr*) blocks the expression of *Slug* without affecting the neural plate. *Fgf8* is expressed in paraxial mesoderm during the time of neural crest cell formation and exogenous FGF8 can exert neural crest cell-inducing function even in the absence of BMP and Wnt signaling (Monsoro-Burq et al. 2003).

Although FGF signaling may represent the first step in the neural crest induction cascade, it is important to note that in *Xenopus* embryos, exogenous FGF8 is unable to initiate expression of the full range of neural crest cell markers induced by the mesoderm. This observation highlights the probable requirement for additional mesoderm factors that act synergistically or in conjunction with FGF8 to generate neural crest cells. Recent results in *Xenopus* embryos confirm this idea. WNT (ectoderm) and FGF8 (mesoderm) signals act in parallel pathways at the neural border and independently converge on *Pax3* activity during neural crest induction (Monsoro-Burq et al. 2005). Despite the substantial evidence of a role for the paraxial mesoderm in neural crest cell induction in frog embryos, it is important to bear in mind that to date there is no evidence to support a similar scenario in fish (Ragland & Raible, 2004) or mouse (P. A. Trainor, unpublished data) neural crest cell induction, nor have assays critically compared head and trunk crest inductive processes.

Migration of neural crest cells?

It was the ability of neural crest cells to emigrate from their site of origin and populate distant peripheral regions that most piqued the interest of early investigators. With the advent of tools for specifically labelling these nomadic mesenchymal cells, the pathways they follow have been well defined in head and trunk regions of many vertebrate species.

A striking feature of cephalic crest cells is the apparent segregation of frontonasal, 1st branchial arch, 2nd arch and 3rd arch populations from one another (e.g. lamprey: Horigome et al. 1999; McCauley & Bronner-Fraser, 2003; axolotl: Epperlein et al. 2000; salamander: Stone, 1929; chick: Noden, 1975; opossum: Vaglia & Smith, 2003; mouse: Trainor & Tam, 1995). This early segregation results from a combination of several autonomous events: (1) the punctuation of the progenitor population by localized apoptosis at the levels of rhombomeres 3 and 5 (Lumsden et al. 1991; Sechrist et al. 1993, 1994; Ellies et al. 2002); (2) the presence of barriers to ventral movement, such as the otic placode, pharyngeal pouches and the optic vesicle, and possibly underlying paraxial mesoderm (Farlie et al. 1999); and (3) the ability of crest cells at the borders of each band to establish cohesiveness sufficient to maintain boundaries (Fig. 6).

This segregation is temporary, and all crest mesenchymal populations subsequently re-establish continuity dorsal and ventral to pharyngeal pouches and around the optic vesicle. The mesenchymal bridges dorsal to each pouch are sites where integration occurs among crest populations located in adjacent branchial arches (Noden, 1983a) and having different *Hox* gene expression histories (Trainor & Krumlauf, 2000a). Also, these are the locations at which profound evolutionary changes have occurred, e.g. the transformations of branchial arch 1 and 2 skeletal elements to middle ear ossicles (Moore, 1981; Martin & Luo, 2005).

Surprisingly, the actual mechanisms by which crest populations and individual cells traverse from their sites of origin to their terminal destinations are poorly understood, especially in the head. Researchers have postulated many underlying mechanisms, ranging from exploitation of paths of least resistance to invasive behaviour to passive population expansion (MacMillan, 1976; Tucker, 2004) by crest cells. However, few analyses of the population dynamics of head crest cells during their dispersive phase have been made, and even basic distinctions between individual cell and population behaviours are lacking (see Locascio & Nieto, 2001).

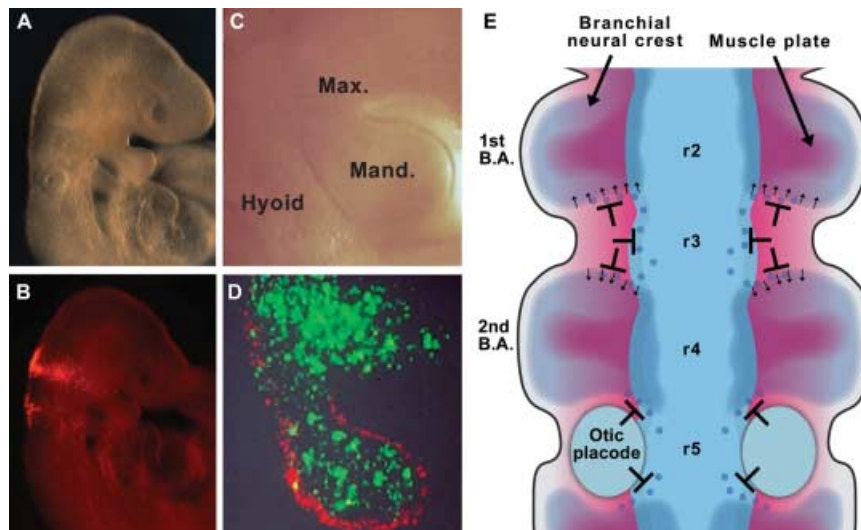


Fig. 6 Segregation of neural crest cells and paraxial mesoderm. (A,C) Low and higher magnification right lateral views showing the head of a 9.5 days post-conception mouse embryo. B is the same as A but illuminated to reveal the locations of rhombomere 2- and 4-derived neural crest cells, which were labelled with a red fluorescent lineage tracer. This illustrates their segregation in streams that colonize the first and second branchial arches, respectively. (D) The same embryo as C with cranial mesoderm cells (green) from the level of the preotic hindbrain occupying the central, presumptive myogenic core of the first branchial arch. These are enveloped by rhombomere 2-derived neural crest cells (red). Other dispersed mesodermal cells are probably angioblasts. (E) Diagrammatic dorsal view summarizing the movements of cranial neural crest populations (blue) and paraxial mesoderm cells (red) into branchial arches. Inhibitory cues (T-bars) restrict lateral movement of neural crest cells (blue shading and blue dots) from rhombomeres 3 and 5, and cohesive forces (arrows) help maintain segregation within branchial arch crest cells.

Conjugates of the verb 'to migrate' are used routinely to describe the behaviour of neural crest cells, including in titles of papers by both authors of this review. We define cell migration as the active movement of cells, alone or collectively, using motility mechanisms to change their location relative to other cells and/or matrix components. For the neural crest, this means that each (or most) individual cell executes filapodial extension and retraction accompanied by directional translocation of the cell body relative to its surroundings, which include the extracellular matrix and neighbouring tissues, both epithelial (surface ectoderm, pharyngeal endoderm) and mesenchymal (paraxial and lateral mesoderm; Kulesa & Fraser, 2000; Teddy & Kulesa, 2004). The robust locomotory capabilities of neural crest cells *in vitro* are well established (reviewed in Bronner-Fraser, 1993; Perris, 1997), although many of these studies in fact document the dispersal of crest cells from high to low cell density environments. Very few direct analyses of the mechanisms underlying early amniote crest cell translocation *in vivo* have been performed.

Transplantation-based mapping studies of avian cephalic crest cells, surface ectoderm and superficial paraxial mesoderm revealed that each population changes position or expands unidirectionally from

dorsal to ventral during the 2 days after cephalic crest cells are produced (Fig. 7; D'Amico-Martel & Noden, 1983; Noden, 1983a,b). These studies prompted the question: do cephalic crest cells migrate actively or move passively by adhering to overlying and underlying tissues, recognizing that the two alternatives are not exclusive?

Neural crest cells that form the enteric nervous system largely arise from the level of the occipital somites, and must undertake the furthest and most prolonged movements. Recent examinations have revealed that single migrating cells are rarely found near the leading edge of this crest population. Rather, the cells collectively form a reticular network of interconnected multicellular cords (Conner et al. 2003; Young et al. 2004). At the leading edge of this expanding reticulum, crest cells are assembled in cords, with only the leading cell extending multiple filopodia. The population expands by progressive proliferation of cells behind the leader of each cord, with continuous formation and remodeling of cross-connected cords behind this advancing front. This process is morphologically identical to that described for embryonic angiogenesis (Noden, 1991c; Risau & Flamme, 1995; Patan, 2000) and, indeed, several molecular similarities have been found (Brantley-Sieders & Chen, 2004; Poliakov et al. 2004).

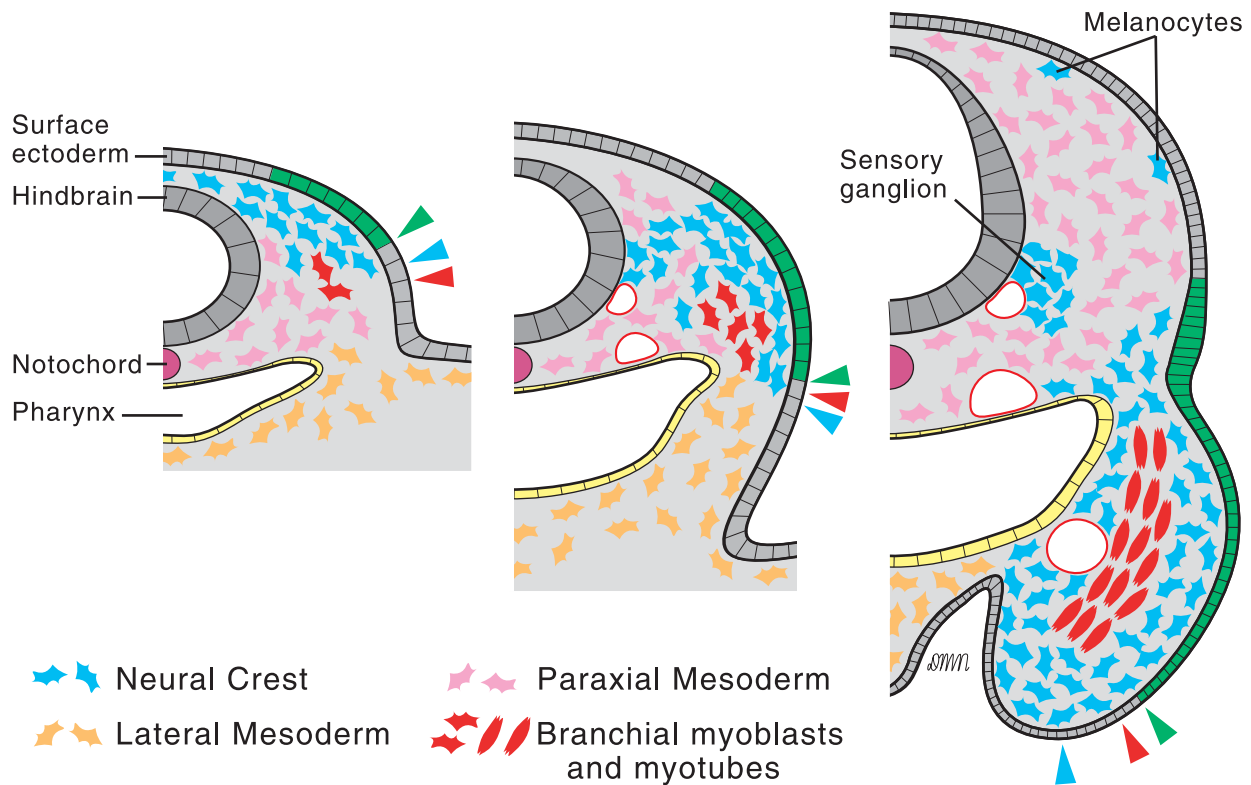


Fig. 7 Schematic transverse views of three stages in the development of the avian head, showing the concerted movements and expansions of surface ectoderm (green), neural crest (blue) and superficial (myogenic) paraxial mesoderm (dark red) populations. Arrowheads show the locations of the leading (ventral) edge of each population. All populations shift and expand in the same dorsal-to-ventral direction during these stages.

Re-examination of previous elegant SEM examinations of 'migrating' trunk (Tosney, 1978; Erickson & Weston, 1983) and cranial (Anderson & Meier, 1981; Tosney, 1982; Nichols, 1986) crest populations reveals an identical but heretofore not appreciated appearance: very few isolated cells are present and multicellular cords extend from the leading edge. This distinction between active cell migration and an expanding, cohesive population with specialized leading edge cells is critical not only to understand better the mechanisms underlying movements of crest populations, but also to refine the explanations for many developmental pathologies collectively referred to as neurocristopathies (Kissel, 1981; Jones, 1990; Nakamura, 1995; Bolande, 1997).

The preceding data and interpretations do not negate traditional modes of cell migration by other neural crest-derived populations, or some members within the large community of amniote cranial neural crest. Certainly, observations on sparse populations of crest cells, for example in the head region of zebrafish

and medaka embryos (Halloran & Berndt, 2003), Schwann cell precursors associated with neuronal processes (Speidel, 1964), and melanoblasts within immature integument (Kelsh et al. 2004) all document extensive motile behaviours of individual crest cells, similar to those seen *in vitro*.

The movements of neural crest cells and mesodermal muscle precursors associated with branchial arches are tightly coupled (Fig. 8; Noden, 1991a; Trainor & Tam, 1995). For example, progenitors of first arch muscles are always associated with crest populations that arise at the same axial level and will also occupy the first branchial arch. These relations are maintained even at later stages when the muscles and their connective tissues may have moved to other parts of the head (Koentges & Lumsden, 1996). Moreover, this congruence extends to cranial motor nerves and precursors of epibranchial placodes, which are neurogenic foci located approximately opposite each pharyngeal pouch (D'Amico-Martel & Noden, 1983; Baker & Bronner-Fraser, 2001; Streit, 2004). This congruence among the several

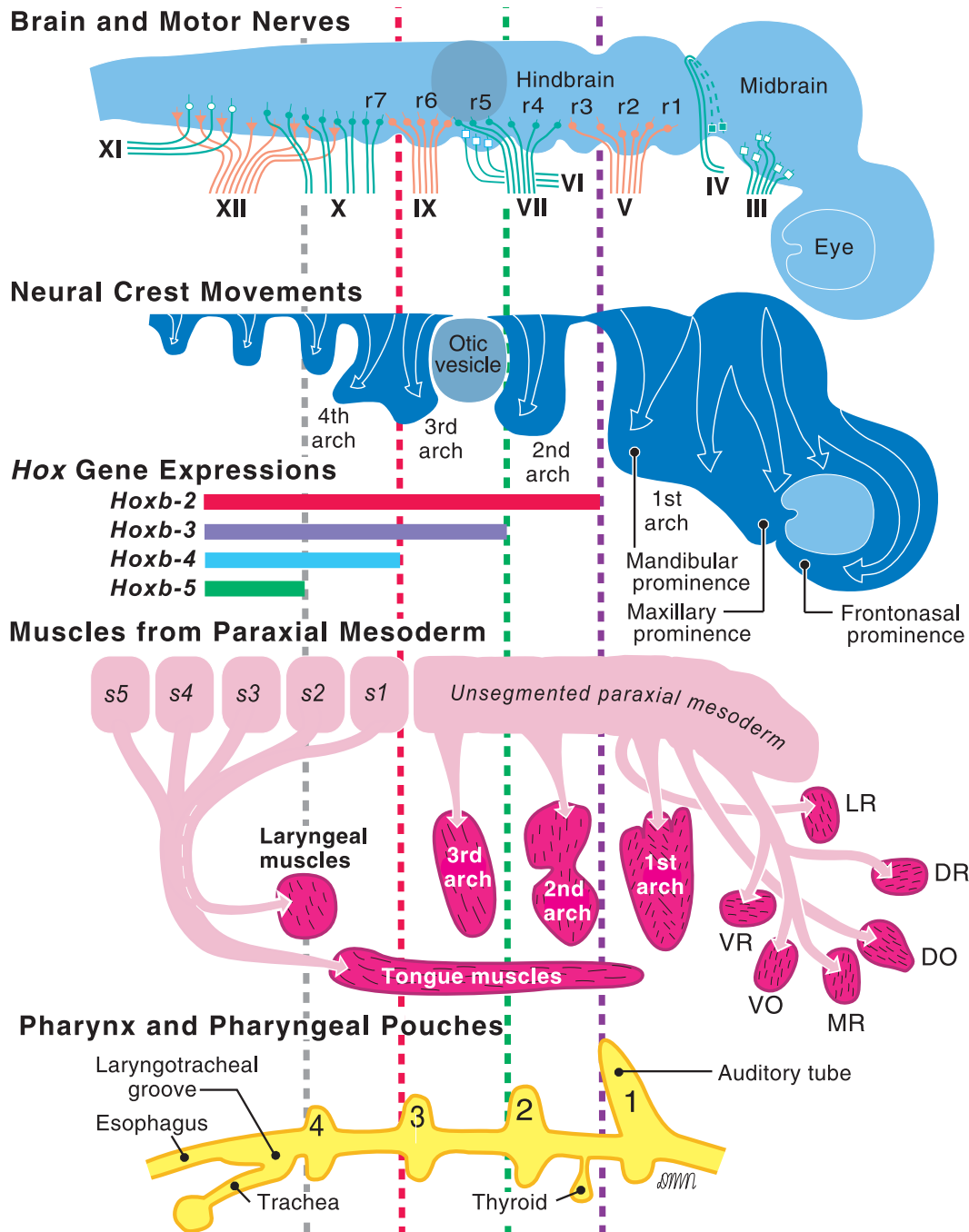


Fig. 8 Staggered, lateral views of all internal tissue layers in an early avian embryo. These illustrate the changes in locations of each population and the spatial relations among them. Neural crest progenitors, cranial nerves and myogenic primordia for each branchial arch all arise at the same axial level and maintain this close registration throughout their dorso-ventral movements. For example, crest cells that will populate the 2nd branchial arch arise from the same axial location (rhombomere 4) as the 7th cranial nerve and the 2nd arch muscles it will innervate. By contrast, the periocular neural crest, extra-ocular muscles and the motor nerves that innervate them all arise at separate axial locations, and do not establish stable relations until all have reached their sites of terminal differentiation.

mesenchymal and epithelial populations destined to occupy each branchial arch allows for ongoing and prolonged interactions that affect the differentiation and spatial organization of each component.

However, registration among key mesenchymal and epithelial populations is unique to the branchial region. Extra-ocular muscles and their crest-derived components arise from disparate axial levels and do not establish

contact until all have independently converged at specific periocular sites. These differences in the initiation of stable neural crest–myogenic mesoderm relations in branchial and periocular regions must be considered in exploring the roles of interactions between them.

The neural crest–mesoderm interface

During the expansion of branchial neural crest populations, long interfaces with underlying mesoderm are established (Figs 1 and 7). In the pharyngeal region, for example, neural crest cells initially colonize the superficial aspects of each branchial arch, and secondarily envelope the mesoderm-derived myogenic core tissues. These extended interfaces between cell populations in the first three branchial arches are probably important for organizing the skeletal, myogenic and endothelial derivatives within the branchial arches (Trainor & Tam, 1995). Except for angioblasts, these interfaces act as barriers to cell movement, at least until later stages of cell differentiation. The molecular basis for preventing cell mixing across the interface is not known. Planes of eph–ephrin boundary have been detected in the head of avian embryos (Kury et al. 2000; Baker & Antin, 2003) and may represent one element of this exclusion.

The tight registration among crest and superficial paraxial mesoderm during the early morphogenetic stages of mesenchymal population translocations suggests that once established this interface remains constant. Although true at the population level, however, these correlative data are not informative with regard to individual cell–cell relations at the interface. Do progenitors of specific muscles and skeletal elements to which they will attach maintain nearest-neighbour relations throughout their development or only come into contact after the extensive dorsal-to-ventral movements are completed? To examine this, cells on both sides of the crest–mesoderm boundary have been labelled during the early stages of population movement, and the locations of their derivatives mapped at later stages (Fig. 9; Evans & Noden, 2005). These results indicate that relations between specific muscle progenitors and their connective tissues are not established until after population expansions and movements are completed. Thus, whereas crest and superficial paraxial mesoderm populations are moving in parallel directions during the same time period, cells on either side of the interface are not positionally coupled.

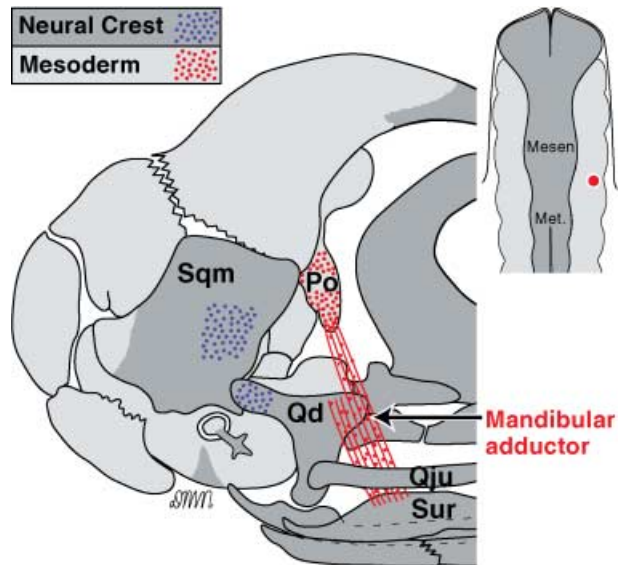


Fig. 9 The loss of nearest-neighbour relations between neural crest and myogenic mesoderm cells is shown. *LacZ*-retrovirus was injected at the interface of these populations, indicated by the red spot on the inset (see also Fig. 1C), and embryos processed 2 weeks later. Mesoderm cells contribute to the cartilaginous postotic (Po) process (red dots) and the mandibular adductor (1st branchial arch) muscle (red lines and dots). However, crest cells that were adjacent to these mesoderm cells while en route did not maintain this nearest-neighbour relation. Rather, as shown by blue dots, they formed osteocytes in the squamosal (Sqm) bone and chondrocytes in the Quadrate (Qd). Thus, while populations remain contiguous during the early, dispersal phases, individual cell relations do not become stabilized until the branchial arch is fully populated.

The crest–mesoderm interface acts as a permanent barrier to mixing of connective tissue precursors. Based on transplantation studies, there are no known situations where cartilage precursors from these disparate mesenchymal populations mix (Noden, 1978a, 1983a; Schneider, 1999). Skeletal structures that are of mixed origin arise due to later fusion of discrete cartilaginous elements, e.g. lateral wings of the basisphenoid derived from crest cells fuse with medial basisphenoid cartilage derived from mesoderm (Noden, 1983a). A possible exception is the avian cartilaginous otic capsule, in which blazes of crest-derived chondrocytes punctuate the largely mesoderm-derived capsule. It has been proposed that these are vestigial branchial elements that have become incorporated into a broad chondrogenic field (Noden, 1983a), and there is little mixing of the two progenitor populations at this interface.

Cranial skeletal muscle is a composite tissue, composed of myotubes from paraxial mesoderm, endothelial cells

also of mesodermal origin, and connective tissues derived from the neural crest, which forms tendons as well as epi- and endomysial tissues. In branchial arch regions, as the paraxial mesoderm and overlying crest populations expand ventrally, crest cells circumscribe the aggregated muscle progenitor population (Schilling & Kimmel, 1994; Trainor et al. 1994; Trainor & Tam, 1995; Cerny et al. 2004). During this process, the muscle is avascular (Ruberte et al. 2003) and remains an exclusively myogenic cord surrounded by neural crest cells. Coincident with the formation and initial alignment of multinucleated myotubes, the surrounding crest cells penetrate the muscle aggregate to establish first the future fascial planes and later delineate individual bundles and muscle fibres (McClern & Noden, 1988).

The situation is different for extra-ocular muscle precursors, some of which (e.g. lateral rectus) are deeply embedded within paraxial mesoderm and do not establish contact with crest cells during the early dispersal stages. Only after the onset of differentiation and aggregation of myoblasts is underway, including the initial formation of elongated myotubes, do these muscle masses shift from their sites of initial differentiation within paraxial mesoderm into periocular mesenchyme derived from the neural crest. This is an extraordinary accomplishment for such a large and cohesive population, and the molecular basis for this translocation is unknown. Borue & Noden (2004) have hypothesized that large, irregular deformations of the mesoderm–crest interface develop, forming finger-like incursions of paraxial mesoderm populations – including eye muscle precursors – deep into crest territory. Newman & Comper (1990) and Newman (2003) have described a process known as matrix-driven translocation that *in vitro* produces comparable patterns of rearrangement of cell populations. In this model, differences in the biophysical properties of the extracellular matrix cause the interface between matrices, and any cells contained therein, to become irregular and tortuous.

Precursors of endothelial cells move invasively and totally disregard the crest–mesoderm interface (Fig. 4). Angioblasts from lateral and paraxial mesoderm actively invade the crest population beginning as soon as crest cells make contact with mesoderm (Noden, 1990, 1991a). Indeed, the 1st and 2nd aortic arches and rostral parts of the cranial cardinal system are in place and patent as soon as the branchial and supra-ocular neural crest populations are in place. These endothelial

cells later recruit surrounding crest cells to form pericytes and tunics of smooth muscle, both peripherally and within those parts of the brain derived from the prosencephalon (Etchevers et al. 2001; Korn et al. 2002). The early cornea and vitreous are invaded by angioblasts, which are subsequently excluded when the anterior and posterior corneal epithelia and the iris differentiate.

Mesodermal influences acting upon neural crest cells

Trunk neural crest cells follow several distinct pathways en route to their final sites of differentiation (Serbedzija et al. 1989; Erickson et al. 1992). Although a few trunk neural crest cells move ventrally within the interface between the somites and the neural tube, most proceed ventrolaterally and contact the dissociating somite. Shortly before somites dissociate into dermamyotome and sclerotome regions, some crest cells arrest their movements, aggregate and initiate the formation of spinal sensory ganglia. Others follow the acellular cleft formed between the basal surface of involuting myotomal epithelial cells and the superficial surface of the sclerotomal mesenchymal population. Crest cells continue moving through paraxial mesoderm environments and upon exiting ventrally they approach, and may contact, the dorsal aorta. A day later, additional trunk neural crest cells begin to migrate dorsolaterally between the ectoderm and somites; these will become pigment cells.

The cranial-to-caudal expanse of each somite is not uniform, with the caudal portion actively preventing further ventral movements by crest cells. As a result, crest cells become concentrated above the cranial half of each somite, creating within the crest a metameric pattern previously present only within paraxial mesoderm (Fig. 10A). Numerous experiments have demonstrated that this restriction of crest cell movement is regulated by inherent properties within the paraxial mesoderm environment. Experimental changes in somite size (Detwiler, 1936), cranio-caudal orientation (Bronner-Fraser & Stern, 1991) or asymmetry (Stern & Keynes, 1987; Kalcheim & Teillet, 1989) have shown that the cranial half of each somite is permissive to neural crest cell entry whereas the caudal half is inhibitory (Fig. 10B–D). Ephrin-B1 synthesis is restricted to the caudal part of each somite, and disrupting Ephrin-B1 ligand signalling permits neural crest cells to enter both the rostral and the caudal halves of the somites

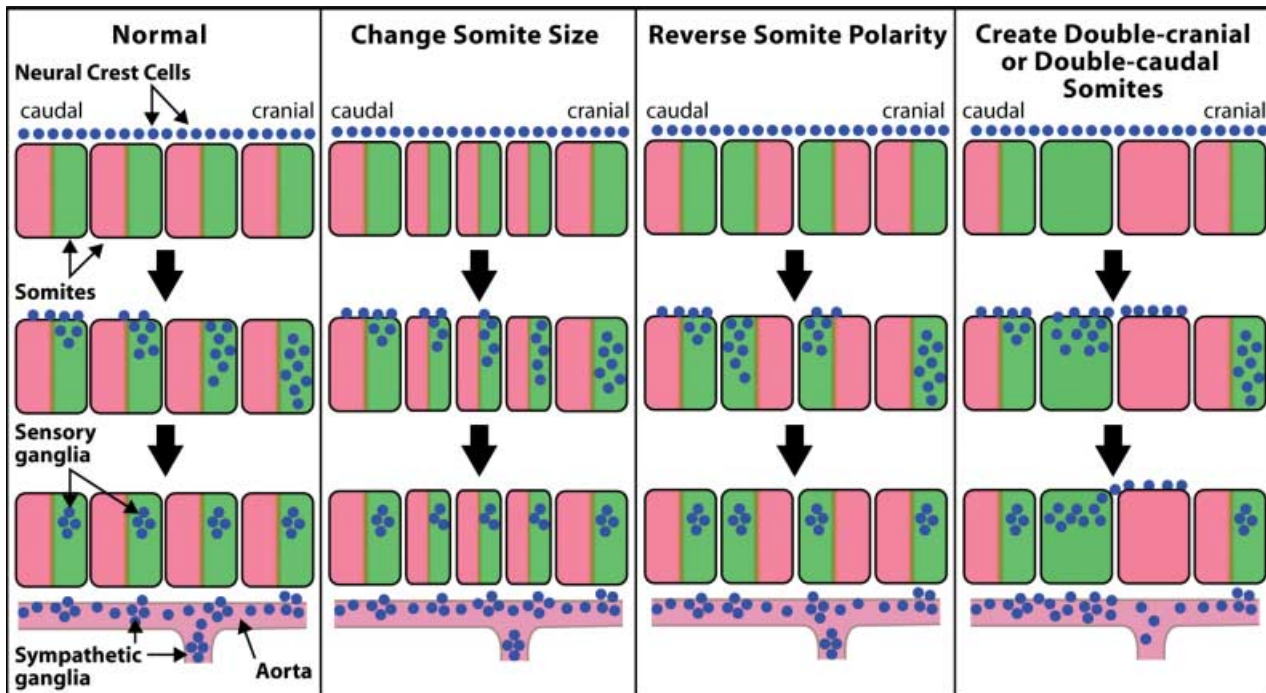


Fig. 10 Schematic lateral representation of the asymmetric, segmental effects that somites exert upon the movements of trunk neural crest cells. (A) The normal responses of crest cells upon contacting the cranial and caudal halves of somites. Surgically altering the size (B), cranio-caudal orientation (C) or cranio-caudal composition (D) of somitic epithelium induces changes in the patterns with which crest cells enter then pass ventrally through each somite.

(Krull et al. 1997). Thrombospondin is found in the cranial half of each sclerotome (Tucker et al. 1999), and may facilitate neural crest cell movements via cooperation with other migration-promoting extracellular matrix components such as fibronectin and laminin.

The regulation of craniofacial neural crest cell movements is more complex and involves interactions with the neural tube, paraxial mesoderm, surface ectoderm and pharyngeal endoderm (Trainor et al. 2002; Cerny et al. 2004; Golding et al. 2000, 2004). Cranial neural crest cells emerge in discrete segregated streams. Each rhombomere has the capacity to generate neural crest cells. However, unlike the rest of the neural tube, most neural crest cells generated from rhombomeres 3 and 5 fail to survive and undergo apoptosis (Graham et al. 1996). Surviving rhombomere 3- and 5-derived neural crest cells move rostrally and caudally to join neural crest cell streams from adjacent rhombomeres (Sechrist et al. 1993; Trainor & Tam, 1995).

Once hindbrain neural crest cells exit the neural tube they are maintained in segregated streams. This suggests that multiple mechanisms must operate to ensure that crest cells reach their correct destinations without inappropriate mixing. Interestingly, if rhombomere

3 or 5 presumptive neural crest cells are transplanted into rhombomere 4, these cells clearly exit the neural tube laterally, mimicking the behaviour appropriate for this location (Trainor et al. 2002). At the level of rhombomere 5 the invaginating otic placode physically restricts the lateral migration of neural crest cells.

Crest cells from midbrain levels grafted into the roof of rhombomere 3 are unable to move laterally, and mimic the normal behaviour of rhombomere 3 crest cells by joining with rhombomere 2 and 4 populations (Noden, 1975). Given the absence of an anatomical barrier at this level, the interface between the ectoderm, mesoderm and neural plate must be essential for restricting lateral cranial neural crest cell migration and also for maintaining streams of neural crest cells as discrete segregated populations for each branchial arch. This process forms part of a conserved mechanism for generating neural crest-free zones that maintain the separation of crest populations with distinct *Hox* expression during vertebrate head development (Trainor et al. 2002).

Upon observing the presence of somitomeres, Anderson & Meier (1981) suggested that these might provide patterning cues to guide crest cells. However,

crest cells are able to change their direction of dispersal. For example, following lesions to local premigratory crest populations, adjacent crest cells readily depart from their normal routes and fill in the depleted areas (Noden, 1978). Paraxial mesoderm in the head does, however, provide at least a permissive substratum analogous to that described above for the cranial half of each somite. Transplanting trunk somites in the place of head paraxial mesoderm partially arrests the ventral expansion of crest populations (Noden, 1986).

This is not exclusively a somite (i.e. epithelial) vs. mesenchymal mesoderm property. Hindbrain crest cells that contact somites 1–3 are prevented from further ventral movements, and instead move rostral to these somites and then proceed ventrally towards the pharynx (Ferguson & Graham, 2004). Replacing somites 1–3 with more caudal trunk somites allows occipital crest cells to mimic trunk crest cell behaviour and move through their new paraxial environment. These results demonstrate that paraxial mesoderm tissues at several sites along the body axis are able to direct (or exclude) crest cell movements independent of their epithelial or mesenchymal organization.

The neural crest as a participant in pattern generation

It is unlikely that there exist any events during head musculoskeletal development in which each progenitor population acts in a fully autonomous or wholly dependent manner; such dichotomies do not exist in embryonic systems. Rather, each participating population brings capabilities and restrictions based on its history. Although there might be hierarchical inequalities in directing the outcome of interactions among the participants, correct histogenesis and morphogenesis require communal interactions. This was first demonstrated by Schotte in his cross-species transplants of mouth-forming tissues (reviewed by Spemann, 1938; updated in Olivera-Martinez et al. 2004b) in which both signal-based and response-based influences were discovered.

Proper morphogenesis of the branchial skeleton requires a complex and progressive interplay between the crest population and its neighbours, both prior to and during their coupled peripheral translocations (Hall, 1991; Trainor & Krumlauf, 2000a; Graham et al. 2003; Trainor et al. 2003). The dominant role of neural crest populations in the development of highly patterned,

branchial arch-specific skeletal structures has been well documented (reviewed by Le Douarin & Kalcheim, 1999; Francis-West et al. 2003; Helms & Schneider, 2003), but often misrepresented. Neural crest progenitors transplanted from one site along the midbrain–hindbrain to a more caudal site will disperse appropriate to their new location (Noden, 1975), but therein will express their original skeletogenic potential, with respect both to tissue types and to their three-dimensional organization (Fig. 11). These results focused attention on the neural crest population as a prominent pattern imposer in the branchial region (Hörstadius & Sellman, 1946; Noden, 1983a), in much the same way as lateral mesoderm acts during limb development.

Comparable results obtained through loss of specific *Hox* gene function in mouse embryos (Gendron-Maguire et al. 1993; Rijli et al. 1993) confirmed the transplantation-based conclusions. The ability of crest populations to acquire and carry axial position-specific morphogenetic information to peripheral locations requires arch-specific combinations of *Hox* gene expression in crest progenitors of branchial arches 2–4, and prior receipt of FGF8-facilitated signals from the midbrain–hindbrain boundary for branchial arch 1 progenitors (reviewed in Rijli et al. 1998; Trainor & Krumlauf, 2000a).

However, both the execution of these early positional specifications and the ability to act cooperatively with crest cells from other sites of origin necessitate ongoing interactions with adjacent tissues. These interactions occur en route as well as at the terminal sites of neural crest cell differentiation. Interactions with paraxial mesoderm (Trainor & Krumlauf, 2000b), pharyngeal endoderm (Couly et al. 2002; Cerny et al. 2004; Le Douarin et al. 2004), and both neural and surface ectoderm (Thorogood, 1993) all modify the execution of prior specifications and are necessary to translate these into the histogenesis and morphogenesis of skeletal tissues.

At sites where crest cells from adjacent arches establish contiguity, cooperativity trumps segmental origin in establishing the outcome. Whenever 1st arch crest cells grafted in place of 2nd arch crest precursors contact neighbouring host crest populations, local interactions drive their development (Fig. 11; Noden, 1983a). This is evidenced also in normal development when individual skeletal elements, e.g. the avian basihyoid, are derived from two initially separate crest progenitor populations (Noden, 1988; Koentges & Lumsden, 1996), and throughout the dermis where adjacent crest

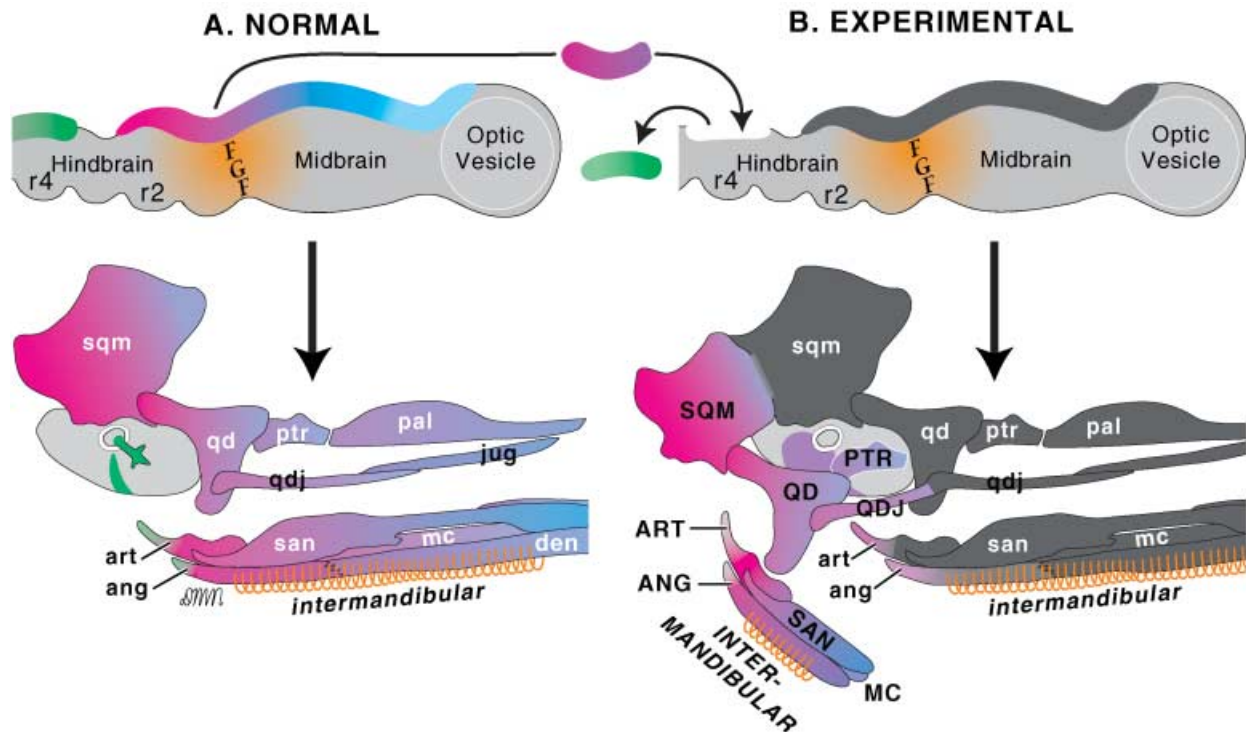


Fig. 11 The morphogenesis of branchial neural crest tissues results from both autonomous, prespecified and dependent, acquired properties. (A) The contributions of crest cells from the mesencephalic (blue, purple), metencephalic (red) and rhombomere 4 (green) levels to the 1st branchial arch skeleton. Note that the proximal (caudal) parts of the articular and angular bones are derived from 2nd arch (rhombomere 4) crest cells that became contiguous with 1st arch (rhombomere 2)-derived crest cells dorsal to the 1st pharyngeal pouch. In B, presumptive proximal 1st arch neural crest precursors (red) were grafted in place of 2nd arch crest precursors. Most formed ectopic 1st arch skeletal structures in the 2nd arch location (labelled in upper-case letters), and directed myogenic cells entering the 2nd arch to form 1st arch-specific muscles, e.g. the INTERMANDIBULAR. At interfaces with neighbouring, untransplanted crest cells, however, grafted crest cells relinquished their prespecified biases and cooperatively formed structures anatomically correct for their new location, e.g. the retroarticular cartilage and proximal angular bone in the 1st branchial arch.

populations or crest and mesodermal progenitors are juxtaposed but leave no evidence of a discontinuity. These outcomes do not negate the importance of early programming of branchial crest populations. Rather, they reveal the importance of dynamic, ongoing interactions with surrounding tissues both to execute historical specifications and to integrate them with other aspects of branchial arch development.

Neural crest cells that circumscribe the prosencephalon become sandwiched between this part of the brain and overlying surface ectoderm. Many of these crest cells form the frontonasal prominences that later, along with the maxillary prominences, establish the midfacial skeleton. The morphogenetic roles of the neural crest in this triad of interacting tissues have been investigated using transplantation, local gene modification and gene knockout approaches. In contrast to the branchial arches, in which the positional

history of crest cells is an essential feature of their morphogenesis, the site of origin of frontonasal crest populations is not a dominant morphogenetic influence (Noden, 1983a). Here, local signals emanating from the prosencephalon and overlying ectoderm are key to patterning of the region (Cordero et al. 2004; Lee et al. 2001; Hu et al. 2003). Crest cells are not passive participants in this process, however, as elegantly shown by cross-species transplantations in which the size, shape and rates of differentiation of midfacial skeletal tissues match those of the crest cell donor species (Schneider & Helms, 2003; Wu et al. 2004; Eames & Schneider, 2005).

Influences of neural crest cells upon paraxial mesoderm

The proximity of crest cells to branchial, but not extraocular, muscle precursors raises the likelihood that

interactions between them might affect the initiation, differentiation and/or morphogenesis of cranial muscles. In chick embryos, expression of the muscle-specific *Myf5* transcript in branchial muscle progenitors is evident by stage 14, which is shortly after these cells have been overlain by crest cells (Noden et al. 1999).

Early evidence from axolotls revealed that when the neural tube was ablated, cranial muscle formation still occurred but the muscles were distorted (Hall, 1999). Unfortunately, it is not clear from this study whether neural crest cell formation, peripheralization or both were affected in the extirpation assay. More recently, specific ablations of cranial neural crest cells were performed in *Bombina orientalis* (Olsson et al. 2001) and axolotl (Ericsson et al. 2004) embryos. Cranial muscles formed but were severely distorted, often lacking attachments and showing abnormal anastomoses with each other. Contacts with cranial neural crest cells therefore appear to be unnecessary for arch muscle differentiation, but are critical for correct branchial muscle morphogenesis in amphibians.

Analyses in avian embryos define a more active role of crest cells in muscle differentiation. Tzahor et al. (2003) found that some of the signals, particularly that of Wnt, that promote myogenesis in somites have instead repressive effects on myogenesis in head paraxial mesoderm. Crest cells that come to overlie branchial muscle precursors produce *Frzb* and *Noggin*, which act as antagonists to Wnt and BMP4 signalling, and thereby release the myogenic population from these differentiation inhibitors. Thus far, this effect has only been documented for 1st branchial arch myoblasts, and the molecular basis for the initiation of muscle-specific gene activation in the head has not been discovered. Moreover, these crest-derived influences are not unique to branchial arch crest populations, as myogenic differentiation proceeds normally even when future periocular neural crest cells are grafted into branchial regions (Noden, 1983a).

For extra-ocular muscles, signals from the central nervous system form part of the muscle-inducing consortium. Segmental plate or newly formed somite tissues from wing levels grafted deep beside the midbrain–hindbrain boundary produce normal dorsal oblique and lateral rectus muscles (Fig. 12), as well as several large, ectopic muscle masses (Noden, 1986; Borue & Noden, 2004). The former differentiate at a slow rate, which is appropriate for head muscles, and express only those genes that are also normally activated in these

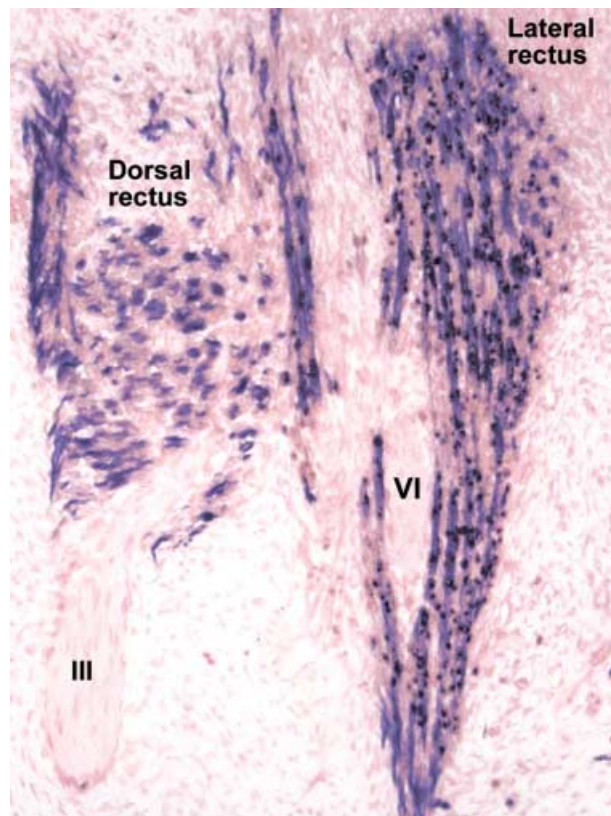


Fig. 12 Quail trunk paraxial mesoderm is able to form normal head muscles. In this micrograph, grafted quail cells (dark, immunopositive nuclei) have formed the lateral rectus but, due to having a different site of origin, not the dorsal rectus muscle. The section was also stained with anti-myosin heavy chain antibodies (blue). This demonstrates both the highly localized inductive properties of head tissues, in this case rhombomere 2, and the ability of periocular neural crest cells to orchestrate the morphogenesis of trunk-derived muscle primordia, which normally would never encounter crest-derived connective tissues. From Borue & Noden (2004).

head muscles. By contrast, ectopic muscles differentiate rapidly, as is appropriate for trunk myogenesis, and express genes characteristic of myotome-derived muscles. As yet, the signals that promote or retard myogenesis of extra-ocular muscles have not been identified.

Although the roles of neural crest cells in muscle differentiation are variable, their profound influence on muscle morphogenesis has been well documented. Within developing branchial muscle masses, the initial formation of elongated myofibres occurs within mesodermal core populations that are surrounded but not yet infiltrated by neural crest cells (Trainor & Tam, 1995). Even at these stages, however, progenitors of individual muscles can be recognized by distinct alignments of myofibres within different parts of the

mesodermal aggregates (McClean & Noden, 1988). Subsequently, after the influx of crest cells between myofibre subsets, individual muscles separate and shift position within each arch (Noden et al. 1999). Experimental modification of the spatial properties of branchial crest cells, due to transplantation or *Hox* gene knockout, consistently and predictably change the patterns of muscle morphogenesis.

Conclusions

Craniofacial development is a complex three-dimensional morphogenetic process during which neural crest cells generate most components of the peripheral nervous system, and the majority of the cartilages, bones and loose connective tissues in the vertebrate facial and branchial regions. Excluding odontogenic cells, paraxial mesoderm forms comparable connective tissues albeit in other parts of the head. Mesoderm also generates progenitors of skeletal muscles and endothelial cells. Proper induction of these lineages in both mesenchymal populations and the subsequent assembly of complex musculoskeletal arrays and their supporting tissues requires progressive and integrated interactions among multiple tissues, including the neuroectoderm, neural crest, surface ectoderm, paraxial and lateral mesoderms, and endoderm. Neural crest cells are neomorphic to the vertebrate head, and it is not surprising that they influence the differentiation and morphogenesis of other tissues, such as the cranial myogenic mesoderm, similar to the roles established for connective tissue progenitors elsewhere in the body.

This review set out to highlight the progressive interactions between neural crest and mesoderm populations during craniofacial morphogenesis. The data available provide good accounts of cell fates and relations, including interactions underlying cell differentiation and morphogenesis within both populations. Largely missing is identification of the signals and interactions that (1) promote contiguity within each population while simultaneously enabling their ventral movements, (2) evoke and promote differentiation among branchial and extra-ocular muscle populations, and (3) direct the assembly of musculoskeletal complexes involving mixed populations.

Craniofacial abnormalities account for up to at least one-third of all congenital birth defects and many of these anomalies are categorized as neurocrestopathies that are thought to arise as a result of perturbations in

neural crest cell formation, distribution (migration?), growth and/or differentiation. The complexity of the interactions that occur between neural crest cells and other cranial tissues during head development demonstrates that many of these craniofacial abnormalities may also occur due to primary defects in the paraxial mesoderm, ectoderm or endoderm tissues with which the neural crest cells interact. Better characterizations of the molecular determinants of pharyngeal endoderm, neural and surface ectoderms and paraxial mesoderm and the effects that signals from each have on neural crest cell development is critical to further our understanding of the origins of congenital craniofacial abnormalities and the basis of craniofacial evolution and diversity.

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